Master's Thesis

Electro-Enzymatic CO_2 Reduction to Formate Using a Formate Dehydrogenase from *Rhodobacter capsulatus*

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Approved by

Advisor
Yong Hwan Kim

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12/05/2018

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Abstract

CO_2 utilization for producing value-added chemicals has recently emerged as a strategy to mitigate atmospheric CO_2 levels. Given that (i) certain formate dehydrogenases (FDHs) are able to interconvert CO_2 and formate, and (ii) formate is versatile in various industries, we, herein, aimed to demonstrate FDH-driven formate production from CO_2. Because of its O_2 stability and being capable of using artificial electron mediators as electron acceptor/donor, we selected formate dehydrogenase from *Rhodobacter capsulatus* (RcFDH), and then constructed a mediated electro-enzymatic CO_2 reduction system. The recombinant RcFDH was expressed in *Escherichia coli* (*E. coli*) and purified under aerobic conditions, and kinetic constants (k_{cat} and K_{M}) for formate oxidation were determined with NAD^+ as electron acceptor. The mediated electro-enzymatic kinetic parameters (k_{red} and k_{ox}) were calculated to optimize the reaction conditions favorable for CO_2 reduction. Finally, the most effective electron mediator and pH conditions were determined by comparison of k_{red} and k_{ox} values, and the RcFDH-driven electro-enzymatic system successfully produced 6 mM of formate in 5 hours.

Contents

List of figures, table, and scheme

Nomenclature

RcFDH: Formate dehydrogenase from *Rhodobacter capsulatus*

SfFDH: Formate dehydrogenase 1 from *Syntrophobacter fumaroxidans*

EcFDH: Formate dehydrogenase H from *Escherichia coli*

MeFDH1: Formate dehydrogenase 1 from *Methylobacterium extorquens* AM1

ARS: Alizarin red S

AQ2S: Anthraquinone-2-sulfonic acid

BV: Benzyl viologen

MV: Methyl viologen

CV: Cyclic voltammetry

FMN: Flavin mononucleotide

bis-MGD: bis-Molybdopterin guanine dinucleotide

KPB: Potassium phosphate buffer

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

NAD^+: Oxidized form of nicotinamide adenine dinucleotide

NADH: Reduced form of nicotinamide adenine dinucleotide

1. Introduction

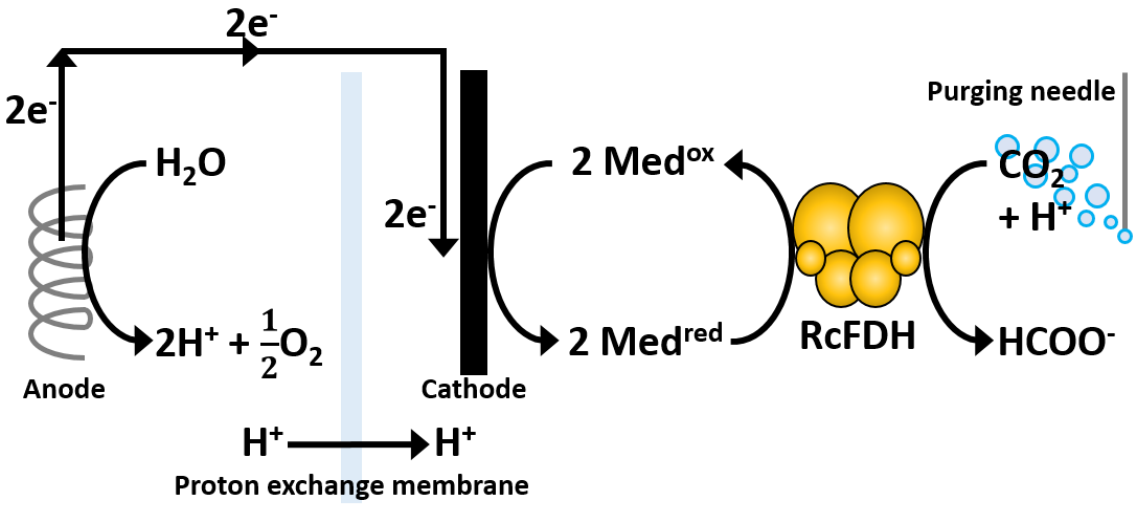
The combustion of fossil fuels has accelerated the accumulation of atmospheric CO_2 and causes climate change and global warming. Thus, recent studies have focused on diminishing atmospheric CO_2 levels via the capture, storage, and utilization of CO_2[1]. Among the strategies, CO_2 utilization is emerging for producing useful fuels and chemicals from CO_2 as a cheap, abundant, and renewable feedstock[2]. Various fuels and chemicals can be obtained from CO_2 (e.g., CO, methanol, and hydrocarbon), and formate is of primary interest because of its versatility. Formate has been used not only in various industrial applications[3] but also as a key platform chemical for further applications such as a carbon source for formatotrophs[4] and fuel for a formate fuel cell[5]. Additionally, Otto et al. assessed the economic and environmental benefits of chemicals obtained through CO_2 conversion and finally formate was selected one of the most beneficial bulk chemicals[6].

Chemical and electrochemical methods have been attempted to convert CO_2 to formate, but practical applications are still limited. CO_2 conversion to formate is usually performed under harsh conditions, and by-products are frequently produced, resulting in low selectivity[7-8]. For example, Azua et al. developed Ir(III)-pincer complex for producing formate from CO_2, but the reaction required high temperature (80 and 200 °C) and high pressure (60 atm)[7]. And Kortlever et al. performed electrochemical CO_2 reduction with copper electrode; formate was mainly produced at -0.85 V (vs. reversible hydrogen electrode), but CO, methane, and ethylene were additionally produced, thereby indicating low selectivity[8]. Given that a biocatalyst usually functions under moderate conditions (i.e., neutral pH, ambient temperature, and atmospheric pressure) and has higher substrate specificity, the enzymatic CO_2 conversion to formate might be a potential alternative.

Although formate dehydrogenase (FDH, E.C. 1.2.1.2) usually catalyzes formate oxidation to CO_2, certain FDHs are capable of reversibly interconverting CO_2 and formate[9-11]. Hirst et al. reported that the tungsten-containing formate dehydrogenase 1 from *Syntrophobacter fumaroxidans* (SfFDH)[9] and the molybdenum-containing formate dehydrogenase H from *Escherichia coli* (EcFDH)[10] are electroactive and can catalyze CO_2 reduction to formate. However, SfFDH and EcFDH function under strict anaerobic conditions, thereby restrictive applications[9-10]. Thus, we focused on FDH from *Rhodobacter capsulatus* (RcFDH), a kind of molybdenum-containing FDH. Hartmann et al. reported that RcFDH is NAD-linked and catalyzes both CO_2 reduction and formate oxidation under aerobic conditions[11]. However, their application for CO_2 utilization seems to be far-off, because CO_2 reduction activities of SfFDH, EcFDH, and RcFDH were just assayed by measuring consumed cofactor and the produced formate might be too marginal to quantify[9-11].

Given that (i) molybdenum-containing FDH is electroactive[10], (ii) RcFDH is O_2-tolerant and expressible in *E. coli*[11], thereby obtaining highly concentrated recombinant protein[11], and (iii) the electro-biocatalytic system is promising for CO_2 utilization to formate[2], we aimed to develop a RcFDH-

driven formate production system utilizing CO_2 and thus constructed by a mediated electro-enzymatic system (Scheme 1). Sakai et al. reported a paper that focused on measurements of the mediated electro-enzymatic kinetic parameters (k_{red} and k_{ox}) between artificial electron mediator and formate dehydrogenase 1 from *Methylobacterium extorquens* AM1 (MeFDH1)[12]. To optimize a mediated electro-enzymatic system favorable for CO_2 reduction rather than for formate oxidation, we calculated the electro-enzymatic kinetic parameters for various pH and electron mediators based on the limiting current measured by cyclic voltammetry (CV). Furthermore, we successfully achieved measurable formate production from CO_2 under aerobic conditions in our optimized electro-enzymatic system.



Scheme 1. Schematic illustration of a mediated electro-enzymatic system for RcFDH-driven formate production from CO_2.

2. Materials and methods

2.1. Expression and purification of recombinant RcFDH

fds operon (5 genes; *fdsG*, *fdsB*, *fdsA*, *fdsC*, and *fdsD*) was synthesized by GenScript (Pscataway, USA), inserted into pTrcHis A plasmid with digestion of *Nhe*I and *Sac*I, and then the recombinant DNA was transformed in *Eshcherichia coli* (*E. coli*) MC1061 (Invitrogen). The mixture of transformed cells was spread on a Luria-Bertani (LB) agar plate containing 150 μg/ml sodium ampicillin. For preculture, one colony was inoculated into 10 ml LB including 150 μg/ml sodium ampicillin, 20 μM isopropyl β-D-1-thiogalactopyranoside (IPTG), 1 mM sodium molybdate, and it was incubated for 12 hours at 37°C without shaking. The LB medium (400 ml in 1 L flask) of same composition as previous medium, in which the 800 μl of cultured cells were added, was incubated for 24 hours at 30°C and 130 rpm. The cells were harvested by centrifugation (4 °C, 4,000 rpm, 40 min) and the cell pellets were stored at -70 °C before use.

For cell lysis, 1 g of the stored cells were suspended in 6 ml of Bugbuster™ (Merck) solution at room temperature for 1 hours with shaking 20 rpm, and then the cell debris was removed by centrifugation (4 °C, 12,000 rpm, 10 min). For purification of RcFDH with N-terminal 6x histidine tag, 1 ml of Ni-NTA agarose resin (QIAGEN) was added to the cell lysate, and the mixture was incubated in ice for 30 min with gentle shaking. The mixture was loaded into the column and the resin was washed with 50 mL of washing buffer (50 mM $NaHPO_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0). The bound RcFDH was eluted with 2 mL of elution buffer (50 mM $NaHPO_4$, 300 mM NaCl, 250 mM imidazole, pH 8.0), and then the buffer was replaced with storage buffer (75 mM potassium phosphate, 10 mM KNO_3, pH 7.5) by Amicon® Ultra centrifugal filters (50 kDa, Merck).

The molecular masses of the FdsG, FdsB, FdsA were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 12 % acrylamide gel under denaturing conditions. All protein bands were visualized using Coomassie Blue (Bio-Rad, Hercules, CA, USA).

2.2. Characterization of RcFDH with UV-vis spectrophotometry

The formate oxidation activity of RcFDH was measured with 2 ml of 100 mM Tris-HCl buffer (pH 9.0) containing 6 mM sodium formate and 2 mM nicotinamide adenine dinucleotide (NAD^+) at 25°C. The reaction was started by addition of enzyme to the above solution, and the activity was calculated by the increase in the absorbance of reduced NAD^+ (NADH) at 340 nm with a UV-spectrophotometer (ε_{NADH}: 6,220 $M^{-1} \cdot cm^{-1}$). One unit was defined as the amount of FDH catalyzing 1 μmol of formate per one minute. In order to determine k_{cat} and K_{M} values for formate oxidation of RcFDH, the activity was measured with a concentration range of 0-4 mM sodium formate by above procedure.

UV-visible absorption spectra of original and reduced RcFDH were measured in 75 mM KPB (pH 7.5) containing 10 mM KNO_3 in a range of 250-800 nm at 25°C. The fully and catalytically reduced RcFDH were prepared by adding 10 mM sodium dithionite and sodium formate, respectively.

For investigation of the stability of RcFDH in acidic and neutral pH conditions, 10 ml of 200 mM KPB of pH range 6.0-7.0 containing 10 mM KNO_3 was used. Stabilization effect of KNO_3 on RcFDH was determined in 10 ml of 200 mM KPB (pH 7.0) with and without addition of KNO_3. In order to identify the relative activity of RcFDH for pH conditions and KNO_3 addition, formate oxidation activity was measured with 100 μl of samples, and initial activity was defined as 100 %.

2.3. Determination of the mediated electro-enzymatic kinetic parameters

All the CV measurements were carried out at 20 mV/s of scan rate in argon-saturated 10 ml of 100 mM KPB containing 100 mM potassium bicarbonate and potassium formate for bicarbonate reduction and formate oxidation, respectively, at 30ºC. Coiled Pt wire, Ag/AgCl (saturated by 3 M KCl) electrode, and glassy carbon electrode (3 mm in diameter) were used as counter, working, and reference electrodes, respectively. 44 μM of alizarin red S (ARS), anthraquinone-2-sulfonic acid (AQ2S), benzyl viologen (BV), and methyl viologen (MV) were tested to determine the most effective electron mediator for RcFDH-driven CO_2 reduction. In order to investigate the optimum pH, CV measurements were conducted with 44 μM MV in 100 mM KPB at pH 6.0-8.0. In the following equations, (i) $k_{\mathrm{cat,red}}$, $k_{\mathrm{cat,ox}}$ and $i_{\mathrm{lim,red}}$, $i_{\mathrm{lim,ox}}$ are the catalytic constants and the limiting currents for bicarbonate reduction and formate oxidation, respectively, (ii) $K_{\mathrm{M,red}}$, $K_{\mathrm{M,ox}}$ and $C_{\mathrm{M,red}}$, $C_{\mathrm{M,red}}$ are the Michaelis-Menten constants and concentrations for the reduced and oxidized electron mediators, respectively, (iii) n_{M}, $n_{\mathrm{HCO_3^-}}$, and $n_{\mathrm{HCOO^-}}$ are the numbers of electrons in the mediator, HCO_3^-, and $HCOO^-$, respectively, and (iv) F, A, D_{M}, and C_{E} are the Faraday constant, the electrode surface area, diffusion constants of the mediator, and concentration of enzyme, respectively[12].

$$k_{\mathrm{red}} \equiv \frac{k_{\mathrm{cat,red}}}{K_{\mathrm{M,red}}} = \frac{(\frac{i_{\mathrm{lim,red}}}{FAC_{\mathrm{M,ox}}})^2}{n_{\mathrm{M}} n_{\mathrm{HCO_3^-}} D_{\mathrm{M}} C_{\mathrm{E}}}$$

$$k_{\mathrm{ox}} \equiv \frac{k_{\mathrm{cat,ox}}}{K_{\mathrm{M,ox}}} = \frac{(\frac{i_{\mathrm{lim,ox}}}{FAC_{\mathrm{M,red}}})^2}{n_{\mathrm{M}} n_{\mathrm{HCOO^-}} D_{\mathrm{M}} C_{\mathrm{E}}}$$

2.4. RcFDH-driven mediated electro-enzymatic CO_2 reduction reaction

The mediated electro-enzymatic CO_2 reduction was performed in an electrochemical reactor, in which the anodic and cathodic compartments were separated by a proton exchange membrane (Nafion®115, DuPont). The anodic compartment was equipped with coiled Pt wire as counter electrode, and filled with 1 mM H_2SO_4 for efficient water electrolysis. A glassy carbon plate (2.0 cm × 1.5 cm) and an Ag/AgCl electrode were placed in the cathodic compartment as working and reference electrode, respectively. CO_2 gas (99.999%) was purged with a flow rate of 100 ml/min into the cathodic compartment, which was consisted of 10 ml of 200 mM KPB, 10 mM KNO_3, 10 mM MV, and 65 U of RcFDH. Formate was quantified by a high-performance liquid chromatography (HPLC, Agilent 1200 series) using an Aminex® HPX 87-H column (300 × 7.8 mm, Bio-Rad) and a refractive index detector[2].

3. Results and discussion

3.1. Determination of expression and activity of the recombinant RcFDH

As previously reported, we successfully obtained a heterologously expressed recombinant RcFDH in *E. coli* and purified it under aerobic condition. It was confirmed by SDS-PAGE analysis, where three bands of structural proteins were appeared with molecular mass of 15 (FdsG), 52 (FdsB), and 105 (FdsA) kDa (Fig.1). FdsA binds a bis-molybdopterin guanine dinucleotide (bis-MGD), five iron-sulfur clusters; FdsB harbors a flavin mononucleotide (FMN) and one iron-sulfur cluster; FdsG binds one iron-sulfur cluster[11]. FdsC acts as a linker for bis-MGD biosynthesis and its insertion into FdsA, and FdsD stabilizes the cofactor transfer from MobA, which converts bis-molybdopterin to bis-MGD, to FdsC[13-14]. Thus, the two proteins are essential to form active holo-RcFDH, but are not structural proteins of the mature RcFDH, so that they were not detected by SDS-PAGE.

The purified recombinant RcFDH exhibited 20 U/mg formate oxidation activity with NAD^+ as the electron acceptor. In order to calculate the amount of catalytically active RcFDH, reduction spectra were recorded as shown in Fig. 2. The UV-visible absorption spectra showed that the overlapping lines at around 418 and 450 nm, which are probably due to the iron-sulfur cluster and FMN cofactor in the RcFDH, respectively. The results revealed that the amount of reduced RcFDH by enzymatic formate oxidation reaction were identical with that by dithionite, thereby indicating that the purified recombinant RcFDH was fully active. When using sodium formate as the substrate, k_{cat} and K_{M} of the purified recombinant RcFDH were determined as 7,440.4 min^{-1} and 237.5 μM, respectively (Fig. 3).

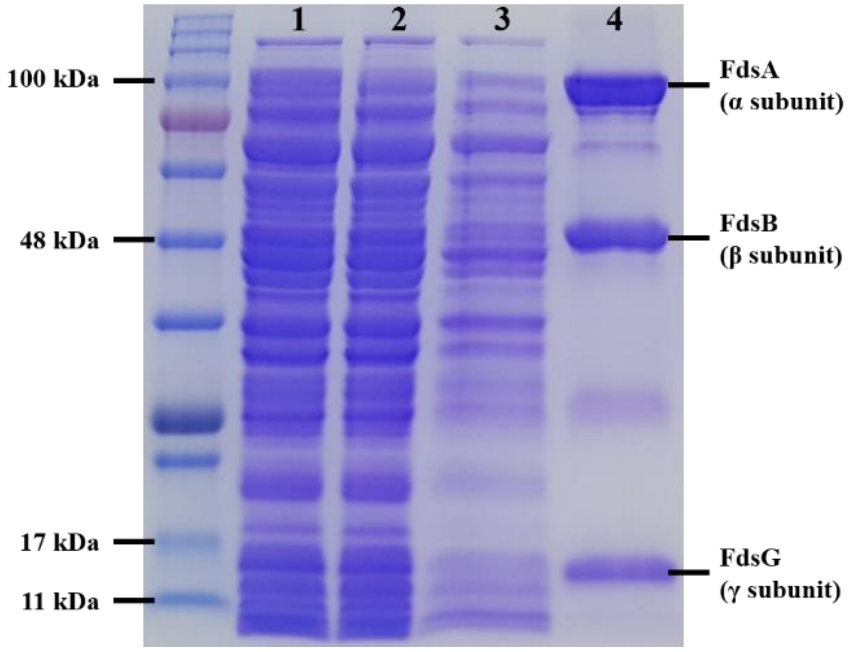


Figure 1. 12% Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) analysis of lane 1: cell lysate, lane 2: flow through, lane 3: washed solution, and lane 4: eluted solution.

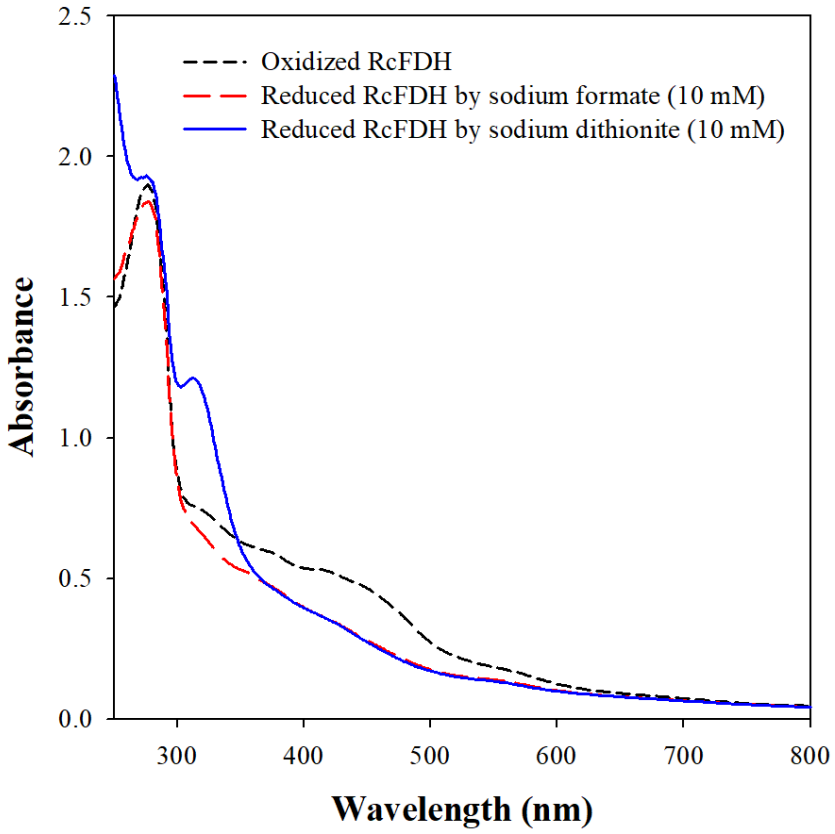


Figure 2. UV-visible absorption spectra of RcFDH. Spectra were recorded in 75 mM potassium phosphate buffer (pH 7.5) containing 10 mM KNO_3 at 25 ºC. The fully and catalytically reduced RcFDH were prepared with the addition of 10 mM sodium dithionite and sodium formate, respectively. The spectra revealed absorption shoulder at 418 and 450 nm, which are probably due to the iron-sulfur cluster and FMN cofactor in the RcFDH, respectively.

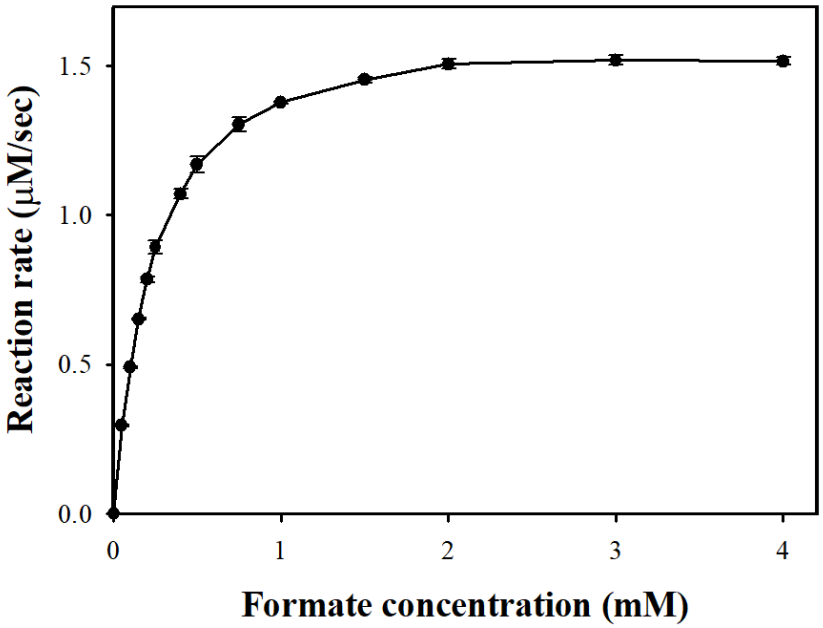


Figure 3. Kinetics of the recombinant RcFDH with 2 mM of NAD^+ at pH 9.0 (100 mM Tris-HCl buffer) and 25 ℃.

3.2. Observation of limiting current in the mediated electro-enzymatic reaction system

Fig. 4 indicates that the reduction and oxidation peak potential of MV were determined at -0.68 V (vs. Ag/AgCl, dashed line in Fig. 4a) and -0.64 V (vs. Ag/AgCl, dashed line in Fig. 4b), respectively. When the formate concentration is much larger than the Michaelis constant for formate, the mediated biocatalytic current attains a limiting value at potentials where the diffusion-controlled reaction of mediator happens, as shown solid lines in Fig.4[15]. The limiting current plateau of the mediated electro-enzymatic bicarbonate reduction and formate oxidation were observed by adding RcFDH at potentials below of -0.7 V (vs. Ag/AgCl, solid line in Fig. 4a) and above of -0.6 V (vs. Ag/AgCl, solid line in Fig. 4b), respectively. Based on the mediated electro-enzymatic kinetic parameters introduced by Sakai et al.[12], we calculated the kinetic parameters of the RcFDH-driven CO_2 reduction ($k_{\mathrm{red}} \equiv \frac{k_{\mathrm{cat,red}}}{K_{\mathrm{M,red}}}$) and the formate oxidation ($k_{\mathrm{ox}} \equiv \frac{k_{\mathrm{cat,ox}}}{K_{\mathrm{M,ox}}}$).

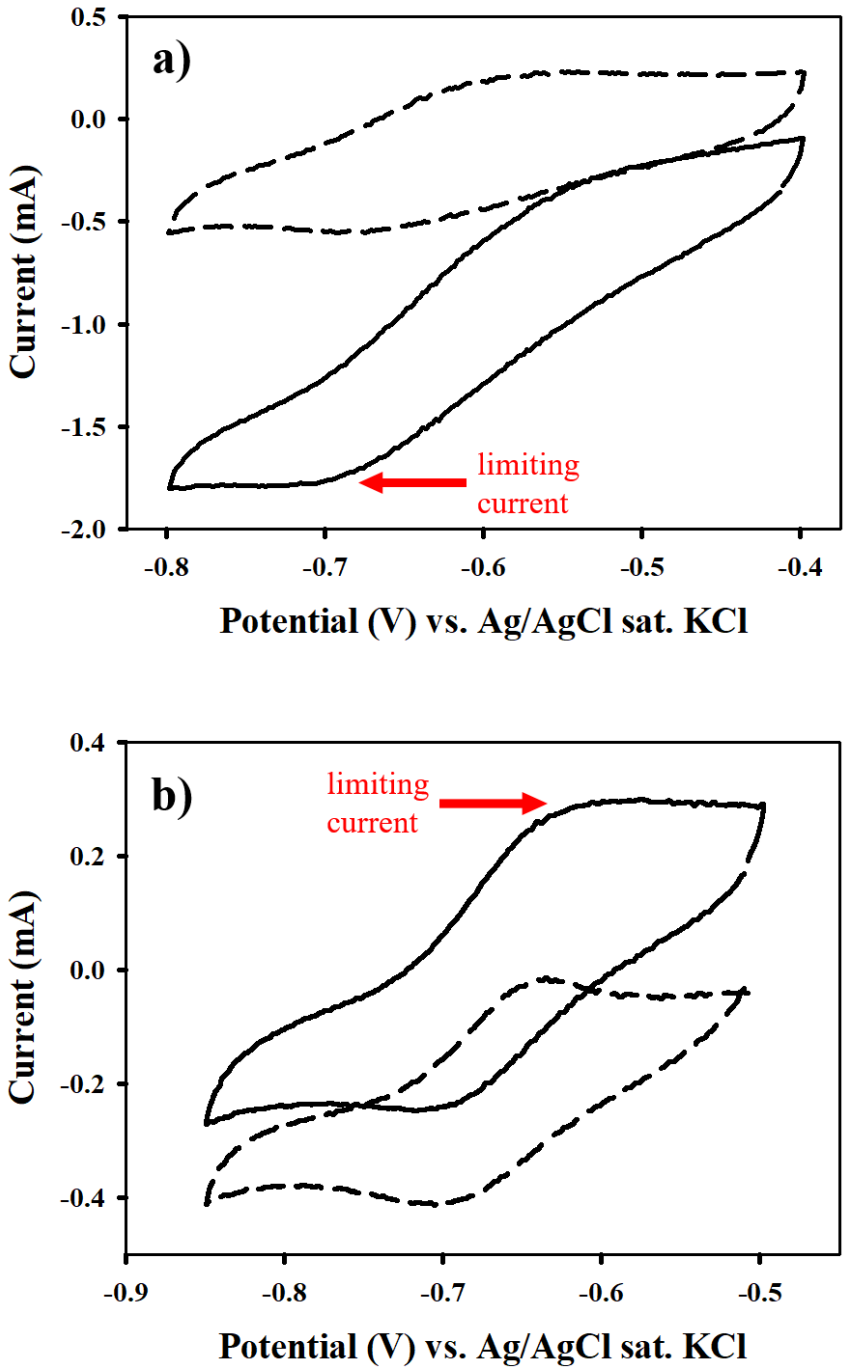


Figure 4. Cyclic voltammograms of 44 μM MV without RcFDH (dashed line), and with RcFDH (solid line) at a glassy carbon electrode in the 100 mM KPB at 30°C (scan rate: 10 mV/s). a) 100 mM potassium bicarbonate reduction at pH 7.0. b) 100 mM potassium formate oxidation at pH 7.0.

3.3. Determination of the most effective electron mediator and pH conditions for RcFDH-driven CO_2 reduction reaction

The mediated electro-enzymatic kinetic parameters between various artificial electron mediators and MeFDH1, which is metal containing FDH such as RcFDH, were determined by Sakai et al. **(11)**. According to their paper, MV, BV, ARS, and AQ2S had lower k_{ox} values or higher k_{red} values for MeFDH1 than other artificial electron mediators. Thus, we tested the four artificial electron mediators to determine the most effective electron mediator for RcFDH-driven CO_2 reduction. Fig. 5a shows that MV was more efficient for CO_2 reduction than other mediators at pH 7.0, and the parameter k_{red} was larger than k_{ox} for MV. Thus, we selected MV as the most suitable electron mediator for the electro-enzymatic CO_2 reduction. Sakai et al. previously reported that a correlation between formal potential of the mediators and $\log k$ values in the MeFDH1-driven electro-enzymatic reaction[12]. In that paper, $\log k_{red}$ was linearly decreased with the formal potential of the mediator, and it was also diminished with the reduction peak potential of the mediator. However, we did not observe a relationship between $\log k_{red}$ and the reduction peak potential (the reduction and oxidation peak potentials of each mediator are summarized in Table 1) probably because of structural differences (e.g., Mo-containing for RcFDH and W-containing for MeFDH1) that might lead to mediator preference under different circumstances[16-18].

We investigated the effect of pH on the mediated electro-enzymatic system. The RcFDH-driven CO_2 reduction was more predominant at an acidic pH, whereas formate oxidation was favorable at a basic pH (Fig, 5b). This phenomenon might be due to the concentrated protons (H^+), which are inevitably required to convert CO_2 to formate, thereby favoring an acidic pH. On the other hand, formate oxidation to CO_2 releases protons and easily occurs at a basic pH. Accordingly, the ratio of k_{red} to k_{ox} (■ in Fig. 5b) implied that pH 6.5 was the most suitable for CO_2 reduction in our mediated electro-enzymatic system. Nevertheless, RcFDH had low stability at an acidic pH, especially at pH 6.0, retaining 81 % of the initial activity after 7 hours (Fig. 6a) and thus RcFDH-driven CO_2 reduction reaction was performed at pH 7.0. In addition, nitrate enhanced the stability of RcFDH (Fig. 6b), thereby 10 mM KNO_3 was employed as the stabilizing additive for the RcFDH-driven CO_2 reduction reaction[19].

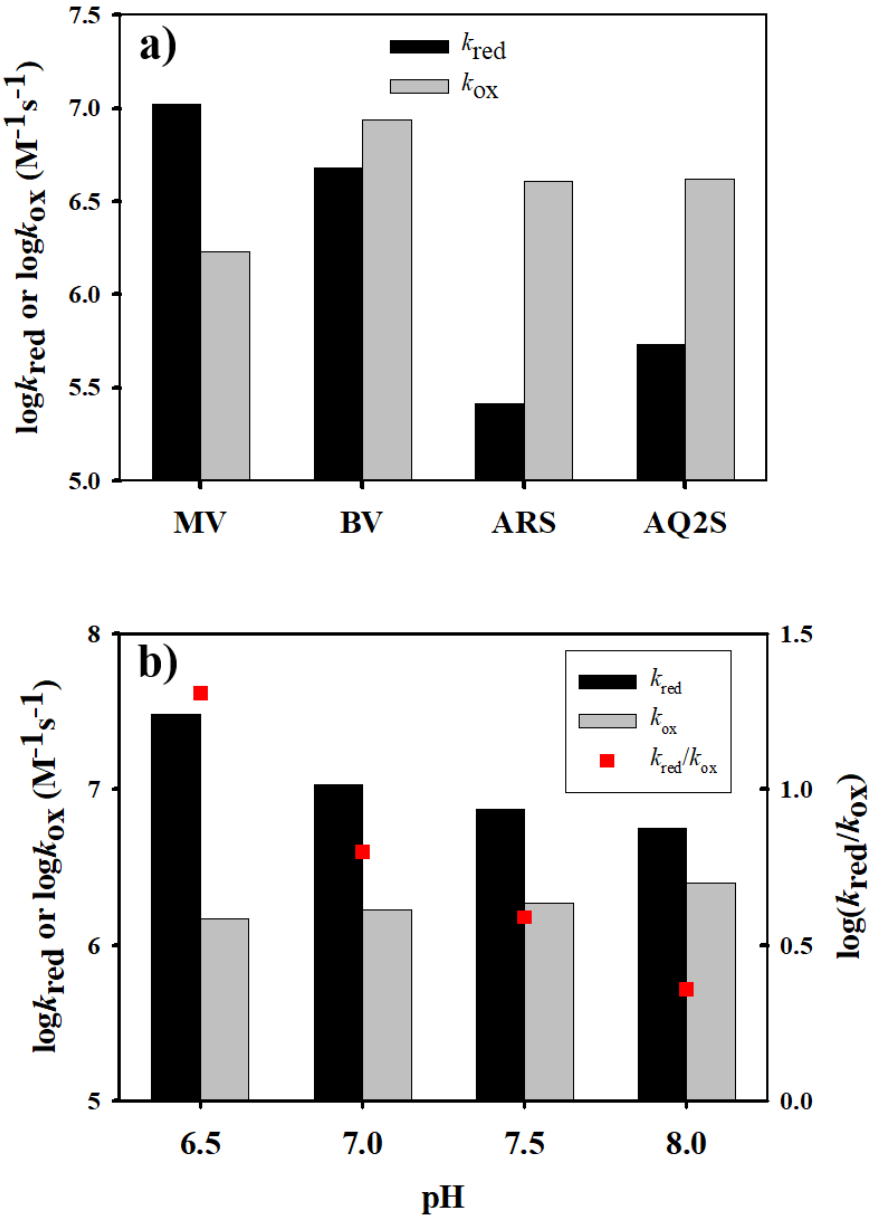


Figure 5. Logarithmic values of kinetic parameters between RcFDH and electron mediators. a) Effect of mediators on the kinetic parameters; MV, BV, ARS, and AQ2S. b) Effect of pH on the kinetic parameters; pH range of 6.5-8.0. $\boldsymbol{k_{red}}$: kinetic parameters of bicarbonate (CO_2) reduction; left *y*-axis (■), $\boldsymbol{k_{ox}}$: kinetic parameters of formate oxidation; left *y*-axis (■), and $\boldsymbol{k_{red}/k_{ox}}$; right *y*-axis (■).

Table 1. Peak potential of electron mediators (100 mM KPB at pH 7.0), potential at which the limiting current was observed, and current of RcFDH-driven CO_2 reduction and formate oxidation for electron mediators at that potential.

Electron mediator	Peak potential (V vs. Ag/AgCl)		CO_2 reduction		Formate oxidation	
	Reduction	Oxidation	Potential (V vs. Ag/AgCl)	Current (μA)	Potential (V vs. Ag/AgCl)	Current (μA)
MV	-0.68	-0.63	-0.71	-1.70	-0.60	0.57
BV	-0.61	-0.44	-0.63	-1.05	-0.42	2.15
ARS	-0.59	-0.55	-0.62	-0.67	-0.54	1.53
AQ2S	-0.48	-0.45	-0.50	-1.19	-0.42	2.04

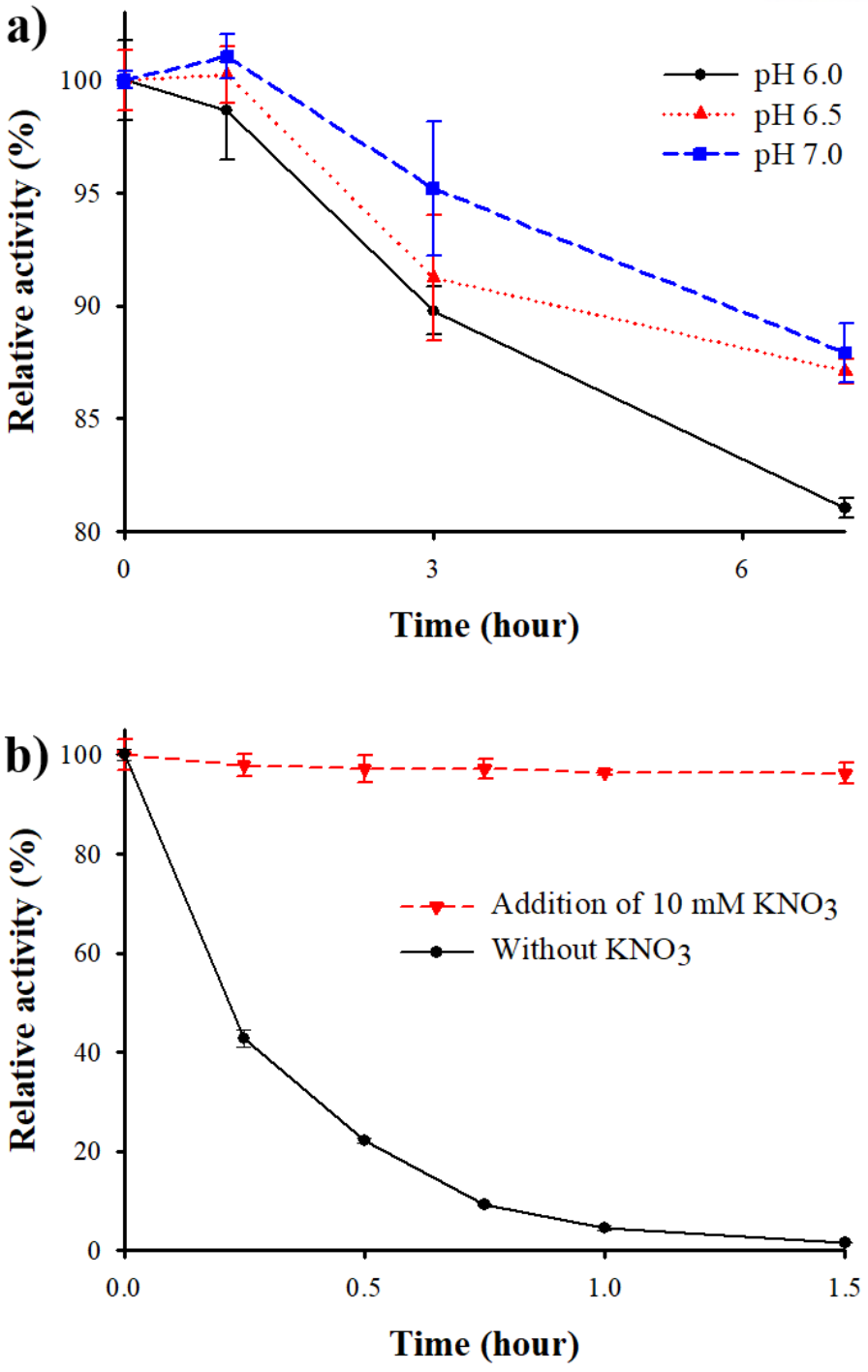


Figure 6. Effect of a) pH (containing 10 mM KNO_3) and b) nitrate addition (pH 7.0) on RcFDH stability (200 mM KPB, 25 °C). The activity measurements of RcFDH were performed as described in Materials and Methods part.

3.4. The mediated electro-enzymatic CO_2 reduction reaction with RcFDH

Finally, RcFDH-driven formate production from CO_2 was performed under optimized conditions with MV as the electron mediator at pH 7.0. In the mediated electro-enzymatic reaction system, first, electron and proton were generated by water electrolysis with Pt wire (anode) in the anodic compartment. The generated proton was supplied to the cathodic compartment via a proton exchange membrane, and oxidized MV (electron mediator) were reduced on glassy carbon (cathode) at -0.75 V (vs. Ag/AgCl). Finally, RcFDH converted the purged CO_2 into formate with the electron from reduced MV and the supplied proton[2]. When using 65 U of RcFDH, we successfully achieved 6 mM of formate production in 5 hours. The current density decreased from initially 0.65 mA/cm^2 to finally 0.55 mA/cm^2, consistent with the decline of formate production rate in Fig. 7, because probably the activity of RcFDH decreased under electrochemical conditions. In an abiotic system as a negative control, no formate production was observed (× in Fig. 7), even though sufficient CO_2 and reduced MV were provided in the reaction solution.

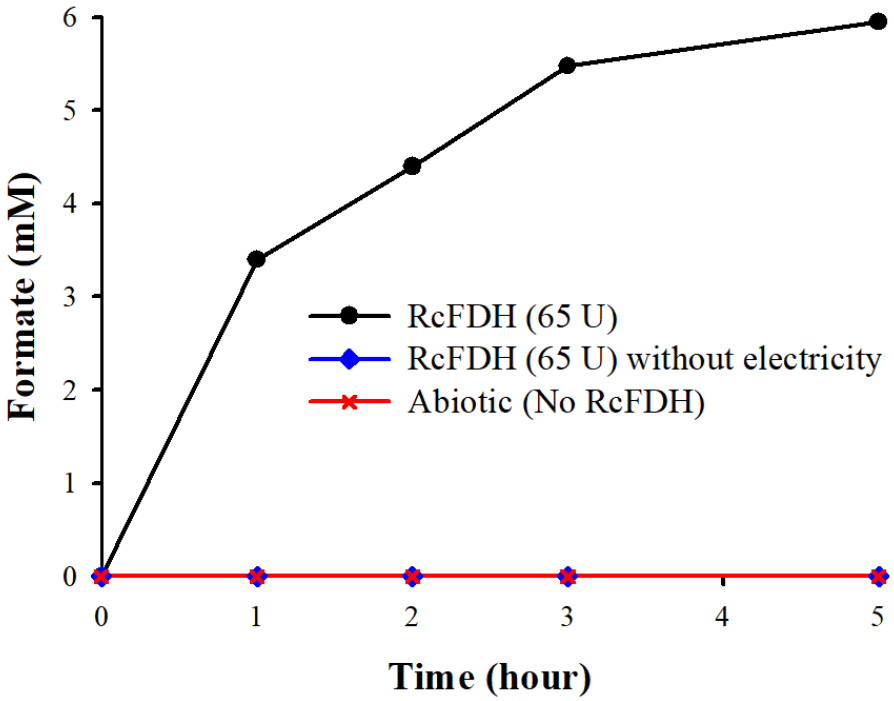


Figure 7. Formate production from CO_2 in the mediated electro-enzymatic system (200 mM KPB containing 10 mM KNO_3, pH 7.0). 10 mM of MV was used as the electron mediator.

4. Summary

Herein, we constructed a mediated electro-enzymatic system for producing formate from CO_2 as a sustainable feedstock. Based on the mediated electro-enzymatic kinetic parameters (k_{red} and k_{ox}), the system was optimized with MV at pH 6.5. Finally, RcFDH-driven CO_2 reduction resulted in 6 mM of formate in 5 hours under aerobic condition. Consequently, the results discussed herein not only might open up the next door to produce the versatile chemical formate from CO_2 as a cheap, abundant, and renewable feedstock but also might contribute to mitigate the atmospheric CO_2 level as a climate change mitigation strategy.

5. Reference

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